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Progress during the report period included: 1) extension of the DEN-1 sequence analysis; 2) mapping of binding sites for anti-DEN-1/JE monoclonal antibodies; 3) development of nonradioactive probes for clinical diagnosis of Dengue; 4) assembly of large clones of DEN-1 cDNA; 5) optimization of procedure for transfection of cells with DEN-1 RNA; 6) investigated ratio between plus- and minus-strand dengue RNA in infected cells; 7) expressed fusion and non-fusion derivatives of DEN-1 proteins in bacterial and eukaryotic systems, including expression via recombinant vaccinia and baculoviruses. Neutralizing antibodies were elicited by E. coli-expressed antigens; neutralizing and protective antibodies were raised in mice against vaccinia-JE recombinants.

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FOREWORD

In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institue of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45CFR46.

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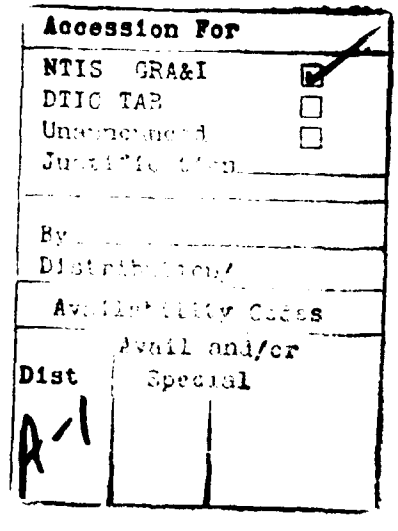

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B. Project Aims

Objectives for the contract period include:

1) to complete functional maps and nucleotide sequences for the genomes of the Japanese encephalitis (JE) and dengue-type 1 (DEN-1) viruses.

2) to develop recombinant viral proteins for potential use as vaccines against the JE and DEN-1 viruses. The proteins of interest are the envelope (E) and non-structural-one (NS1) proteins. Emphasis has been divided between *E. coli* host-vector expression systems and production of recombinant DEN-1 and NS1 immunogens in insect host cells using recombinant viruses of the *Autographica californica* nuclear polyhedrosis virus (baculovirus).

3) analysis of the immunological properties of recombinant E and NS1 proteins and characterization of the vaccine potential of the recombinant proteins. Characterization includes mapping antibody binding domains on the individual proteins and evaluation of protective potential as candidate subunit vaccines. Immunogenicity in mice is assessed by evaluation of titer and specificity of antibodies reacted *in vitro* with authentic E and NS1 proteins, and by potential to induce protection against lethal viral challenge.

4) development of cDNA and RNA probes for use in research and clinical diagnosis.

5) identification and assembly of large clones of DEN-1 cDNAs using PCR amplification of viral RNA.

6) defining conditions to measure the infectivity of DEN-1 genomic RNA and derivatives synthesized *in vitro*.

7) development of sensitive hybridization assays to determine the ratios of plus- and minus-stranded RNAs in DEN-1-infected insect cells.

C. Program

Functional mapping of the JE genome

We continued to use immunological procedures for the identification of viral-encoded polypeptides in JE-infected cells. JE cDNA fragments were expressed in *E.coli* as *trpE*-JE fusion proteins. The expression vector used was designated pATH and is available in all possible reading frames. The resulting fusion product consists of a 37 kDa amino terminal segment of the bacterial *trpE* protein followed by the viral peptide at the carboxy end. As with the *lacZ* fusion products characterized earlier, antibodies raised against these recombinant proteins are useful probes for specific segments of the viral polyprotein.

In collaboration with Dr. Peter Mason, currently at the Plum Island Animal Disease Center of the USDA and assistant professor at Yale University, we continued our analysis of the NS1 protein produced in virus-

infected cells. Antibodies against a *trpE*-NS1 fusion protein were used in Western blots and radioimmune precipitation (RIP) experiments to detect NS1 and a longer form, NS1', in insect (C6/36) and mammalian (Vero) cells infected with the Nakayama strain of JE. Two forms of NS1 were also detected in the extracellular fluid from cultures of infected Vero cells, the smaller form being much more abundant. Dr. Mason has extended this work in his own lab and has used label-chase experiments to examine a possible precursor-product relationship between the NS1 and NS1' proteins. No evidence of such a relationship was found for the JE/Nakayama-infected Vero cell lysates and cell supernatants (Mason, P.W., 1989).

Antibodies against fusion proteins were also used to identify proteins translated from the NS4 and NS5 regions of the JE genome. Immunoprecipitation and western blot analyses revealed the presence of a 29kDa protein that appears to be specified by the NS4a region of the JE genome. In addition, sequences related to this 29 kDa polypeptide appear to be present in a much larger polypeptide, possibly a higher molecular weight form of NS5.

Sequence analysis of the JE and DEN-1 genomes

Approximately 75% (8.1 kb) of the DEN-1 genome was sequenced during the contract period. The sequenced region begins at a continuous open reading frame specifying the capsid (C), membrane (M), and envelope (E) structural proteins and extends to approximately nucleotide 8500 in the NS5 coding region. The sequence of the 3745 nucleotides at the 5' end of the open reading frame has been published in a paper entitled "Sequence of the dengue-1 virus genome in the region encoding the three structural proteins and the major non-structural protein NS1" (Mason *et al.*, 1987b). In this paper, the sequences were compared with corresponding proteins for seven other flaviviruses, including two of the three remaining dengue serotypes, DEN-2 and DEN-4.

A number of minor discrepancies and gaps in the sequence of the region between nucleotides 6500 and 8500 were not resolved before the departure of the guest scientists primarily responsible for the sequencing effort at that time. As our vaccine efforts were concentrated on the E and NS1 proteins, for which our sequence data was complete, priority was shifted away from sequencing during the latter stages of the contract period. This shift in emphasis enabled the project team to work in a more expeditious manner on production of recombinant E and NS1 proteins for epitope mapping and imunogenicity analyses.

Mapping of antigen binding domains of the JE envelope protein

Expression of JE cDNA in *E.coli* using the bacteriophage λgt11 vector led to the identification of two separate regions of the E protein that were efficiently expressed as JE-β-galactosidase fusion proteins and reactive with antibodies present in polyclonal hyperimmune mouse ascites fluid (HMAF). The fusion protein derived from one of these regions, corresponding to amino acid residues 280 to 414 of the E protein, also reacted with 10 monoclonal antibodies (MAbs) generated against antigens expressed in JE virus-infected mice. These 10 MAbs appear to recognize different epitopes on the E protein based on competitive binding analyses (D.S. Burke, personal

communication), cross-reactivity with other flaviviruses, and neutralization titers.

To map the antigenic determinants for these MAbs on the E protein, we isolated the cDNA fragment from the shortest immunoreactive λgt11 clone and ligated it into the polylinker region of the appropriate reading frame pATH expression plasmid. Large quantities of E antigen were expressed from the resulting plasmid in the form of a *trpE* fusion protein. Deletions were generated by *Bal31* exonuclease digestion. The truncated fusion proteins expressed by the deleted plasmids were then used in immunological tests to define the borders of the minimal sequence required for reactivity with the MAbs and HMAF. The epitopes recognized by HMAF and all 10 MAbs map to a region within amino acid residues 303 and 396 of the E protein. This sequence includes two cysteine residues known to form a structurally important disufide bridge in the E protein of flaviviruses, and one of these residues (Cys-304) lies at the N-terminal border of the shortest immunoreactive sequence. Chemical reduction and alkylation studies with the authentic viral protein confirmed the importance of disulfide bridges in the formation of this antigenic structure. These results show that epitopes for at least some strongly neutralizing MAbs lie within a 94 amino acid stretch of the E protein sequence. Furthermore, the presentation of these epitopes apparently requires the formation of a disulfide bridge between Cys-304 and Cys-335. These results were published in Mason *et al.*, 1989.

Mapping epitopes of recombinant DEN-1 E and NS1 antigens.

DEN-1 E and NS1 protein coding sequences were expressed in *E.coli* by subcloning two large fragments of cloned cDNA into appropriate pATH expression vectors. A 1236 bp fragment coding for the first 412 amino acid residues of the 496 amino acid E protein and a 1184 bp fragment specifying the 355 C-terminal residues of NS1 plus 40 amino acids of NS2a were fused in-frame with the bacterial *trpE* gene in pATH-11 and pATH-1, respectively. The fusion proteins expressed by these plasmids cross-reacted with antisera and MAbs against DEN-1. Of 60 MAbs tested, five reacted with the E-*trpE* fusion and five others reacted with the NS1-*trpE* fusion. The known properties of these MAbs are given in Table 1.

DNA deletion analysis was used to define the minimal sequences necessary to express the antigenic determinants for these antibodies. For mapping the C-terminal ends of the antigenic regions, we first linearized the vector through random cutting with DNase I and then inserted a *Bam*HI linker. After identifying plasmids with linker inserts within the viral cDNA, the new restriction site was used to delete downstream coding sequences. This method produced a series of 19 nested deletions for the *trpE*-E protein with an average size increment between clones of approximately 70 bp. Similarly, 12 incremental deletions of about 100 bp were obtained in the NS1 sequence. For creating N-terminal deletions, the sequences downstream from the linker insertion were subcloned back into pATH vectors to establish new in-frame fusions to *trpE*. Precise mapping was accomplished by *Bal31* exonuclease digestion of selected plasmids.

The immunoreactivity of the truncated fusion proteins was examined by ELISA and western blotting. Two antigenic regions have been mapped in the E protein sequence (see Figure 1). Domain I contains the epitopes for two

Table I. Properties of monoclonal antibodies reactive to recombinant DEN-1 antigens expressed in *E.coli*.

MAb[a]	Group[b]	Protein specifity[c]	PRNT[d]	Cross-reactivity[e]
8C2	I	E	nd	type
8B9	I	E	nd	subcomplex (D1, D3)
9D12	II	E	>10,000	type
4E5-6	II	E	>10,000	flavivirus subgroup
13D-4	II	E	nd	flavivirus subgroup
13B-6	I	NS1	nd	type
13A1	I	NS1	nd	flavivirus subgroup
15F3	II	NS1	nd	subcomplex (D1, D4)
5C11	II	NS1	nd	type
7E11	II	NS1	nd	subcomplex (D1, D4)

[a] The monoclonal antibodies are described in Gentry *et al.* (Amer. J. Trop. Med. Hyg.(1982) 31, 548-555), Henchal *et al.* (Amer. J. Trop. Med. Hyg. (1982) 31, 830-836) and Kaufman *et al.* (Amer. J. Trop. Med. Hyg. (1987) 36, 427-434).refs.(1-3) Two of the MAbs 9D12 and 4E5-6, bind to topographically related sites.
[b] The antibody groups were defined by the 3' deletion series.
[c] Protein specificity was determined by western blot analysis with the native viral antigens.
[d] PRNT; plaque reduction neutralization titer.
[e] The cross-reactivity determined by either western blot or ELISA.

MAbs and lies between amino acid residues 76 and 93. Domain II contains epitopes for three MAbs, at least one of which (4E5-6) confers passive protection in mice. This domain lies between residues 293 and 401 (109 aa) and contains an essential disulfide bridge. Although the N-terminal border of this region has not been precisely determined by *Bal31* digestion, an analogous determinant on the E protein of JE has an N-terminal border at residue 303. The NS1 protein was mapped by the same strategy and the results obtained for the deletion variants are shown in Figure 2. Two overlapping domains have been identified. The first is between residues 57 and 104 (48 aa), the second between residues 81 and 125 (45 aa). These mapping results are described in Mason *et al.*, 1990.

Immunogenic properties of the JE fusion proteins.

Collaborators at WRAIR examined the protective potential of the three JE *trpE*-E/NS1 fusion proteins shown in Figure 1. All of the proteins induced a strong antibody response in mice. But, disappointingly, the antisera lacked detectable virus neutralizing antibody and none of the proteins provided protection against virus challenge. At the same time, a positive immunization control consisting of the Nakayama killed virus vaccine (Biken) did provide good protection. Possible bases for the lack of

protection by the recombinant antigens include interference by the adjoining *trpE* moiety and/or absence of immunologically correct conformers. (As indicated above, maintenance of particular disulfide cross-links is essential for the binding of our test neutralizing antibodies in *in vitro* assays).

Development of non-fusion DEN antigens.

The negative results obtained with the JE fusion proteins caused us to modify our strategy for producing recombinant immunogens in *E.coli*. Since that point the focus of our synthetic vaccine effort with *E.coli* was directed at synthesizing non-fusion proteins, with very little or no 'foreign' peptide. In the initial strategy selected, flavivirus proteins were expressed from a vector in which viral cDNA is fused to a plasmid segment containing a bacterial ribosome binding element, a translational start signal and codons for only six or seven 'foreign' amino acids. Transcription of the relevant mRNA is mediated by the strong, inducible P_L promoter of phage lambda, via heat inactivation of a thermolabile repressor.

Figure 3 shows the structure of the variable reading frame (vrf) vector series used for expression of non-fusion variants of DEN-1 E and NS1. The coding regions selected for expression encode amino acids 238-413 and 293-413 of E and 57-153 of NS1. These regions include the major epitopic domains of each immunogen expressed in *E.coli*, as determined by our earlier epitope mapping analyses. The E protein variants also contain a segment that corresponds well with a 9-kDa proteolytic fragment of the tick borne encephalitis virus E protein previously shown to elicit neutralizing antibodies in mice (Heinz *et al.*, 1984). These domains also contain fewer cysteine residues than do the full-length proteins, which reduces the potential for forming aberrant disulfide bridges. The E protein vectors shown are derivatives of constructs that originally allowed translational readthrough into adjoining non-viral RNA sequences. This problem was remedied by subsequent engineering of a translational stop at the end of the viral cDNA region.

Good expression results were obtained for the E protein vectors. Under inducing conditions, the viral protein comprises upwards of 15% of the total protein in the recombinant organisms and the products correspond in size to that expected for a non-fusion construct. An example of the protein pattern for the longer E recombinant protein, designated Δ34, is shown in Figure 4. Results from western blot assays showed this protein (residues 238-413 of E, which includes Antigenic Domain II), to be recognized by both polyclonal and monoclonal antibodies. Most encouraging was the observation that this derivative is recognized by virus neutralizing monoclonal antibody.

Effective expression and purification schemes were developed for producing biochemical quantities of the Δ34 protein. A flow diagram showing the purification steps is provided in Figure 5. The procedure is simple, involving: i) disruption of the bacterial cells and concentration of the recombinant protein by centrifugation (the protein product self-associates to form an insoluble aggregate), ii) solubilization of the protein in the presence of a denaturing agent followed by extraction in an acid solution condition, iii) dialysis in urea and a sulfhydryl reducing agent - to cleave possible intermolecular disulfide bonds, iv) purification by chromatography

on a carboxymethyl cellulose ion exchange resin, and v) dialysis to remove urea and reducing agent - to permit protein renaturation. This procedure has been scaled up successfully to yield about 50 milligrams of protein per five liters of culture.

The CM cellulose column profile shown in Figure 6 reveals excellent separation of the main contaminant(s) from Δ34. Subsequent electrophoresis of the peak-two-protein on SDS-polyacrylamide gels, followed by Coomassie blue staining reveals a single homogeneous band of the molecular size expected for Δ34 (Figure 7). The purified Δ34 is recognized by polyclonal and monoclonal antibodies in both immunoblots and Elisa assays, including monoclonal antibodies shown to have virus-neutralizing activity.

Further characterization of Δ34 was undertaken to evaluate the extent of internal disulfide bond formation. A two-step alkylation procedure was used to modify: i) free and ii) total cysteines (i.e. free plus S-S-bonded). The state of modification--and, thus, disulfide bonding--was determined by gel electrophoresis (Hirose, *et al.*, 1988, Anal. Biochem., 168, 193-201). With this strategy, we determined that purified Δ34 contains one disulfide bond, analogous to the situation that obtains for the corresponding domain in authentic DEN-1 E protein (Figure 8).

Immunogenicity of the Δ34 DEN antigen

The immunogenic potential of the Δ34 antigen was evaluated at WRAIR by Dr. Hoke and Mr. J. Mckown, with disappointing results. Like the earlier JE fusion proteins, this product elicited a strong immune response in mice. However, the resulting antisera did not contain detectable neutralizing antibody and no protection was apparent in a virus challenge assay.

Subsequent to that experiment, a variant of the Δ34 antigen was constructed via site-directed mutagenesis. As is illustrated in Figure 8, the Δ34 protein contains three cysteine residues, two of which--cys_{302} and cys_{334}--are believed to be involved in formation of a disulfide bridge which is required for antibody recognition. In the new construct, the potential for formation of the wrong disulfide bond--an event that could possibly result in loss of immunogenicity--was eliminated by replacing cys_{285} with a serine residue. The altered antigen is known as Δ34 cys-to-ser, or simply Δ34-ser. We further altered our expression protocol by placing the coding regions of both the original Δ34 and the Δ34-ser proteins into plasmid pET3c, one of the T7 RNA polymerase expression vectors constructed by Studier and co-workers (Studier *et al.*, 1990). This vector is designed for easily inducible, high-level expression of recombinant proteins by the T7 RNA polymerase. A third DEN-E antigen, Δ31--spanning amino acids 293-413-was also placed in plasmid pET3c. All three proteins were expressed at high levels in this system, representing approximately 20% of total cell protein. The proteins were visualized by coomassie blue staining and by immunoblotting, using the virus neutralizing antibody, D2-9D12. (It is noteworthy that neither Δ31 nor Δ34-ser were detectable when expressed in the previously used λPL system.)

Several experiments were performed to test the immunogenicity of the Δ34 and Δ34-ser antigens in mice. In the experiments presented below, the antigens were bound to alumina immediately prior to injection into the

animals. Immunization occurred on days 0, 14, and 28, followed by injection of sarcoma cells on day 35 in order to generate ascitic tumors. Sera from the mice were characterized for anti-DEN-1 antibody titers by plaque reduction neutralization tests (PRNT), western blot analysis, hemagglutination inhibition and ELISA. The results for the PRNT assays are presented below in Table 2.

Table 2. Plaque reduction neutralization titers for mouse antisera generated by immunization with *E. coli*-produced recombinant DEN-1 proteins.

ANTIGEN	ANIMAL	PRNT[1]
Δ34-SER (ALUMINA)	1	nd[2]
	2	1:20
	3	nd
	4	nd
	5	nd
	6	nd
	7	nd
Δ34-CYS (ALUMINA)	1	1:40
	2	1:40
	3	nd
	4	1:40
	5	1:20
	6	1:20
	7	1:40
	8	1:80

[1] Dilution of antiserum yielding greater than 50% plaque reduction
[2] nd = no plaque reduction observed at 5-fold dilution

The data presented in Table 2 are significant for several reasons. First, the recombinant antigen produced in bacteria elicited virus neutralizing antibodies. The PRNTs for the Δ34 protein were 5- to 10-fold lower than the values obtained with ascitic fluids generated with hybridoma cells secreting the neutralizing antibody D2-9D12 (data not shown). We believe these results represent the first demonstration of dengue neutralizing antibodies generated with a recombinant antigen produced in *E. coli*. Second, alumina appears to offer significant advantages over complete Freund's adjuvant (CFA) for generating neutralizing antibodies with recombinant antigens. in several previous immunization trials over a period of two years, we were unable to detect neutralization titers in any of the animals immunized with recombinant antigens in CFA. The basis of the beneficial effect of the alumina is not obvious. However, one possibility is that adsorption of the protein to the surface of alumina particles is an effective way of presenting the antigen in the "correct" conformation. The results also show that the removal of cysteine residue 285 in the E protein sequence actually decreased the effectiveness of the Δ34 antigen. As noted above, we originally thought that the presence of cysteine-285 might interfere with the formation of the essential disulfide bridge between cysteines 302 and 333 in the E protein sequence (Mason *et al.* 1989, 1990).

The antisera to the Δ34 protein were reactive in western blots against the authentic E protein form DEN-1 infected cells, but the titer of these sera was very low in hemagglutination inhibition assays (little or no inhibition at 1:5 dilution). By comparison, the HAI titer for the D2-9D12 monoclonal antibody was greater than 1/4096.

Recombinant E and NS1 proteins: Non-bacterial expression systems

Efforts were initiated to assess the potential of producing effective recombinant immunogens in eukaryotic cells, where maturation and folding of the virus protein products may occur more naturally than in an *E. coli* system. Some of these efforts were carried out in collaboration with other groups, as is noted below.

Expression of JE proteins from recombinant vaccinia viruses

Work with vaccinia vectors was initiated by our group, but the major effort was in collaboration with Dr. Enzo Paoletti of the Virogenetics Corp. and Dr. Peter Mason of Yale University. Very encouraging results have been obtained, as reported in Konishi *et al.*, 1991 and Mason *et al.*, 1991. Recombinant vaccinias were identified that correctly specified the synthesis, glycosylation and secretion of NS1, and the synthesis and glycosylation of the E protein. Immunization of mice with recombinant viruses resulted in immune responses to NS1 and E, and the viruses provided full protection against lethal challenge with JEV. Further, the high level of protection was correlated with the production of high titers of neutralizing and hemagglutination-inhibiting antibodies. It is of considerable interest that the tested viruses were found to produce an extracellular hemagglutinin containing M and E that sediments on sucrose gradients similarly to the hemagglutinin found in the culture fluid of JEV-infected cells. It is speculated that the particle produced by the vaccinia recombinants represents an empty JEV envelope in which the E and M proteins are folded and assembled into a "natural" configuration, probably explaining the ability of the recombinant virus to induce a high level of neutralizing antibodies.

Baculovirus-mediated expression of DEN-1 proteins

Using the cDNA library described in Mason *et al.* 1987, a 5′ segment of the DEN-1 genome was cloned into the *Autographa californica* nuclear polyhedrosis virus transplacement vector pVL941. Three recombinant baculoviruses expressing recombinant DEN-1 proteins were isolated. The best characterized of these, BR-7, contains the coding sequences for C, prM, M, E and 80% of NS1 (the carboxy-terminal 70 amino acids of NS1 are replaced by 33 amino acids encoded by the polyhedrin gene of the vector).

Positive results were obtained when cells infected with BR-7 were fixed and subjected to an immunofluorescence assay that used an anti-DEN-1 hyperimmune mouse ascites fluid (HMAF), an anti-NS1 HMAF and monoclonal antibodies 9D12 and 7E11, directed against E and NS1, respectively. Immunoblotting performed on BR-7-infected cell extracts using the 9D12 antibody revealed that the recombinant E protein produced by these cells comigrates with E from DEN-1-infected mosquito cells. A truncated form of

NS1 was also detected in these extracts using the 7E11 antibody. All of the tested antisera identified proteins that were larger than E or NS1 in the BR-7-infected cell extract; these are presumed to be either unprocessed polyprotein or partially or improperly processed polyproteins. In addtion, antisera produced in the immunized mice were evaluated by immunoblotting. The antibodies were found to react with authentic E and NS1 from DEN-1-infected C6/36 cells, and with DEN-1 antigen in extracts of DEN-1-infected BHK cells, suggesting that DEN-1 proteins produced in the baculovirus system are capable of illiciting an immune response that recognizes authentic DEN-1 proteins. These results are described in Huebner *et al.*, 1990.

The immunogenic potential of the BR-7 antigen was further evaluated by determining the plaque reduction neutralizing titers of the antisera raised in mice injected with total protein extracts from baculovirus-infected Sf21 cells. Two immunization protocols were tested: the protein was injected as an emulsion with Freund's complete adjuvant, or, alternatively, the protein was mixed with a suspension of alumina prior to injection. Typically, animals were immunized with one of the antigen preparations on days 0, 14 and 28 of the experiment, followed by injection with sarcoma cells on day 35 in order to generate ascitic fluid. The data from these experiments was disappointing; the maximum PRNT observed was 1:20, and in most cases was lower than that (PRNT = dilution of antiserum yielding greater than 50% plaque reduction).

Development of sensitive nucleic acid probes for use in clinical diagnosis

The aim of this work was to establish protocols for the production of highly sensitive nonradioactive RNA or DNA hybridization probes for use in detecting and discriminating between virus nucleic acid in test materials, a subject of special importance for technical workers in the field and clinical personnel. Results were obtained from side-by-side comparison of DEN-1 probes tagged with biotin by three different enzymatic methods: i) *in vitro* transcription of cDNA using T7 and T3 phage RNA polymerases; ii) nick-translation of cDNA fragments; iii) random primer labeling of cDNA. For labeling of RNA transcripts, allylamine-UTP was used as a substrate, and the RNA was biotinylated in a second reaction with CAB-NHS (caproylamidobiotin-N-hydroxysuccinamide ester). Biotinylation of the DNA probes was accomplished by incorporation of biotin-21-dUTP, a dTTP analog with biotin attached to the pyrimidine ring by a 21-atom spacer arm. Comparison of the probes in dot blot assays using alkaline-phosphatase-conjugated avidin for detection showed that the RNA probes were the most sensitive, followed by random primer labeled DNA and, finally, nick-translated DNA.

Hybridization analyses were performed using the labeled RNA and a variety of target RNAs including those from uninfected and DEN-1 infected C6/36 cells and a DEN-1 virion preparation. Results indicate that less than 5 pg of DEN-1 RNA from virions can be detected using this method and a positive signal can be detected with less than 50 pg of RNA from DEN-1 infected cells. No background signal was detected with a 10,000-fold excess of RNA from uninfected cells.

Identification and assembly of large clones of DEN-1 cDNAs using PCR amplification of viral RNA

Recombinant DNA technology is being employed to examine the antigenic potential of recombinant proteins produced in prokaryotic and eukaryotic expression systems. A logical progression in the use of genetic engineering in vaccine development is the production of recombinant attenuated viral strains. This can be achieved through the construction of full-length DEN-1 cDNA for transcription into infectious RNA molecules.

The strategy developed for the production of full-length DEN-1 cDNA involved the use of the polymerase chain reaction to amplify segments of the genome that can be ligated together and then transcribed *in vitro* to produce infectious RNA. Two fragments (fragments #2 and #3--see Figure 9), representing sequence information from nucleotides 2580-7235, have been amplified and cloned into plasmid pBluescript SK-. These fragments correspond to 80% of the coding sequence of NS1 through the amino terminal 50% of NS4b. The 5'-terminal segment, corresponding to D-1 nucleotides 1-2579, was amplified but could not be cloned into pBluescript SK-, perhaps due to the presence of an extra A residue added to the 3' end of amplified products by the terminal deoxyadenosine transferase activity of Taq DNA polymerase. The 3'-segment, beginning at nucleotide 7236, was not amplified by the PCR stragegies employed. This may indicate a failure of the consensus primers to initiate first strand synthesis. Two possible reasons for this are: 1) the secondary structure at the 3' end of D-1 RNA might inhibit first strand synthesis, or 2) the consensus sequence used to design primers 4Aa, 4Ab, and 4Ac may differ from the actual sequence of the D-1 RNA (see Table 3).

Optimization of conditions to measure the infectivity of DEN-1 genomic RNA and derivatives synthesized in vitro

The evaluation of *in vitro*-synthesized RNAs requires an efficient procedure for transfection of the RNA into susceptible host cells. To this end, we have optimized conditions for transfection with DEN-1 RNA extracted from virions. Prior to this study, the only flavivirus nucleic acid used for transfection experiments was yellow fever RNA (Rice *et al.*, 1989). The results of the present study indicate that DEN-1 RNA can be successfully transfected into cultured insect cells. This procedure should make it possible to assay the infectivity of transcripts prepared both *in vivo* and *in vitro*.

The following conditions were used to obtain efficient transfection of BHK-21-15 cells with DEN-1 RNA. Approximately 1.5×10^5 cells in a 177 mm^2 area (one well of a standard 24 well culture plate) were transfected with 5 mg lipofectin and up to 1 ng full-length DEN-1 RNA in the presence of 500 ng yeast carrier tRNA. Thus, the lipofectin-RNA ratio was approximately 10:1. Lipofectin-RNA incubations were performed at 37°C with 500 ml Opti-MEM per well. An incubation time of seven hours resulted in transfection efficiencies in the range of 21-36 PFU/ng. This is approximately 5-30 fold less than the transfection efficiency obtained by Rice *et al.* (1989) for yellow fever RNA. It may be that a longer incubation (up to 24 hours) would result in an increased transfection efficiency.

Table 3 Oligonucleotide primers

Oligonucleotide[1]	Sequence	Tm (°C)[2]	%GC	Notes
1S	GCG-GCC-GCA-TTT-AGG-TGA-CAC-TAT-AAG-TTG-TTA-GTC-Twx-GTG-GAC [3]	56-60 [4]	40	- SP6 promoter - NotI site[5] - 21 nucleotide consensus
1A	GAT-TCC-ACT-AAC-GTC-TCC-TA	58	45	- primer: nucleotides 2661-2670 of D-1 sequence - XhoI site: 2579
2S	TAA-GAG-ACT-ATC-AGC-GGC-CA	60	50	- primer: nucleotides 2509-2528 of D-1 sequence - XhoI site: 2579
2A	CGG-TTC-AAC-AGC-AAT-CAC-CT	60	50	- primer nucleotides 4798-4779 of D-1 sequence - BamHI site: 4751
3S	AGA-GAC-TGG-AAC-CAA-GTT-GG	60	50	- primer nucleotides 4680-4699 of D-1 sequence - BamHI stie: 4751
3A	CAT-CGT-AAA-CCA-CAG-GGT-CC	62	55	- primer nucleotides 7280-7261 of D-1 sequence - SalI site: 7235
4Sa	CTG-ACG-CTG-ACA-GCG-CGG-TA	66	65	- primer nucleotides 7110-7129 of D-1 sequence - SalI site: 7235
4Sb	GCT-AGT-GGC-TCA-TTA-TGC-CAT-AAT-TGG-AC	84	45	- primer nucleotides 7135-7163 - SalI site: 7235
4Aa	ACT-AGT-CGG-ACC-GAG-AAC-CTG-TTG-wyT-CAA-CAw-C	58-66[4]	49	- RsrII site - SpeI site [5] - may be mismatch near 3' due to JE sequence
4Ab	GAG-CAG-TAC-TAG-TCG-GAC-CGA-GAA-CCT-GTT-GAT-TCA-ACA-GCA-C	66[4]	51	- 23 nucleotide consensus - RsrII site - SpeI site - ScaI site
4Ac	GAG-CAG-TAC-TAG-TCG-GAC-CGA-GAA-CCT-GTT-GAT-TCA-ACA-GCA-CCA-TTC-CA	86[4]	43	- 30 nucleotide consensus - RsrII site - SpeI site - ScaI site
A5SP	GTC-CAC-wxA-GAC-TAA-CAA-CTT-ATA-GTG-TCA-CCT-AAA-TGC-GGC-CGC			annealed to primer 1S to test SP6 promoter

1. S = sense strand sequence A = antisense strand sequence

2. Tm = 4 (G+C) + 2 (A+T) (Innis and Gelfand, 1990)

3. lowercase letters indicate a mix of nucleotides as follows: w = G and A x = T and C y = A and T

4. Tm based on consensus sequence (denoted by boldface type)

5. Restriction site engineered for primers 1S (NotI site) and 4Aa (SpeI site) do not contain a sufficient number of surrounding nucleotides for efficient digestion. Therefore, when these sites are used, linkers must be added.

Determination of the ratios of plus- and minus-stranded RNAs in DEN-1-infected insect cells

Experiments were initiated to accurately determine the relative levels of "plus" and "minus" strand DEN-1 transcripts present in infected C6/36 cells at various time-points, post-infection. Total cellular RNA was extracted from cultures grown from 1 to 5 days following infection with DEN-1. Various amounts of RNA--ranging from 0.1 μg to 50 μg--were hybridized to excess ^{32}P-labeled, *in vitro*-transcribed, 1-kb RNA probes complementary to either plus or minus DEN-1 transcripts. The hybridization reactions were subsequently treated with ribonuclease T1, which cleaves single-stranded RNA molecules at guanosine residues, but which does not recognize double-stranded RNA structures. Thus, probe that was hybridized to a plus- or minus-strand DEN-1 transcript was protected from enzymatic digestion. Separation of the reaction products on a denaturing polyacrylamide gel showed clearly that a single band, 1 kb in length, was protected from RNase T1 activity in both the "plus" and "minus" reactions. As ribonuclease protection assays (RPA) of this sort are performed using radiolabeled probes of known specific activity, the levels of the protected probes--and, by inference--levels of the corresponding DEN-1 transcripts--can be determined following separation of the reaction products on a denaturing polyacrylamide gel. Our preliminary results indicate that, under the infection conditions used in this set of experiments, the ratio of plus-strand to minus-strand transcripts five days after initial infection of the culture is at least 10:1, and probably much higher. Extremely low levels of minus-strand transcripts in all of the samples made precise quantitation difficult.

Our results indicate that the RPA approach is potentially a useful method for quantification of *in vivo* levels of DEN-1 transcripts, but several modifications to the infection protocol used in the preliminary experiments are required. First, it is necessary to infect the cultures under conditions that ensure simultaneous infection of virtually all of the cells. Experiments performed subsequent to the RPA tests discussed above indicate that a 100% simultaneous infection rate requires a multiplicity of infection of approximately 10. To meet this requirement, we prepared high-titer stocks of DEN-1 by two methods: concentration of existing low-titer stocks by ultracentrifugation; and by preparation of new stocks using a modified infection protocol. Second, we have refined our definition of early-, mid- and late-infection time-points by determining, via immunofluorescence, that DEN-1 antigens can be detected on the surface of 100% of the C6/36 cells 36 hours after infection at a high multiplicity of infection, whereas no immunofluorescence can be detected at 24 hours post-infection. Thus, future RPA experiments should be performed on total cellular RNA extracted from cells infected at a high multiplicity of infection, at several time-points ranging from a few hours to 36 hours after infection. We believe that simultaneous infection of all cells in the culture will significantly improve the yield of DEN-1 specific transcripts in total cell RNA preparation, thus solving the problem of low absolute levels of minus-strand RNA.

D. REFERENCES

Dieckmann, C.L. and Tzagoloff, A. (1985) Assembly of the mitochondrial membrane system. CBP6, a yeast nuclear gene necessary for synthesis of cytochrome b. J. Biol. Chem. *260*:1513.

Gentry, M.K., Henchal, E.A., McCown, J.M., Brandt, W.E. and Dalrymple, J.M. (1982) American J. Trop. Med. Hyg. *31*:548.

Heinz, F. X., W. Tuma, F. Guirakhoo, R. Berger and C. Kunz (1984) Immunogenicity of tick-borne encephalitis virus glycoprotein fragments: epitope-specific analysis of the antibody response. J. Gen. Virol. *65*:1921-1929.

Henchal, E.A., Gentry, M.K., McCown, J.M. and Brandt, W.E. (1982) American J. Trop. Med. Hyg. 31:830.

Henchal, E.A., Narupiti, S., McAda. P., Fournier, M.J., Mason, T.L., Padmanabhan, R., Blok, J., Hoke, C. and Nisalak, A. (1986) Rapid detection of dengue and Japanese encephalitis viruses using nucleic acid hybridisation. *In*: Arbovirus Research in Australia: Proceedings Fourth Symposium. St. George, T.D., Kay, B.H. and Blok, J. (eds.) CSIRO Tropical Animal Science, pp. 112-118.

Huebner, R.C., Wysokenski, D., Mason, T.L. and Fournier, M.J. (1990) Expression of antigenically active Dengue-1 virus proteins using recombinant baculovirus. Vaccines 90: 125-130.

Kaufman, B.M., Summers, D.R., Dubois, D.R. and Eckels, K.H. (1987) American J. Trop. Med. Hyg. *36*:427.

Konishi, E., Pincus, S., Fonseca, B, Shope, R., Paoletti, E. and Mason, P.W. (1991) Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. Virology *185*:401-410.

Mason, P.W., Dalrymple, J.M., Gentry, M.K., McCown, J.M., Hoke, C.H., Burke, D.S., Fournier, M.J. and Mason T.L. Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. (1989) Journal of General Virology *70:2037-2049*.

Mason, P.W., McAda, P.C., Dalrymple, J.M., Fournier, M.J. and Mason, T.L. (1987a) Expression of Japanese encephalitis virus antigens in *Escherichia coli*. Virology *158*:361-372.

Mason, P.W., McAda, P.C., Mason, T.L. and Fournier, M.J. (1987b) Sequence of the Dengue-1 virus genome in the region encoding the three structural proteins and the major nonstructural protein NS1. Virology *161*:262-267.

Mason, P.W., Pincus, S., Fournier, M.J., Mason, T.L., Shope, R.E. and Paoletti, E. (1991) Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. Virology *180*:294-305.

Mason, P.W., Zuegel, M.U., Semproni, A.R., Fournier, M.J. and Mason, T.L. (1990) The antigenic structure of Dengue Type 1 virus envelope and NS1 proteins expressed in *Escherichia coli*. Journal of General Virology *71*:2107-2114.

McAda, P.C., Mason, P.W., Schmaljohn, C.S., Dalrymple, J.M., Mason, T.L. and Fournier, M.J. (1987) Partial nucleotide sequence of the Japanese encephalitis virus genome. Virology *158*:348-360.

Nowak, T. and Wengler, G. (1987) Analysis of disulfides present in the membrane proteins of the West Nile flavivirus. Virology *156*:127.

Rice, C.M., Grakoui, A., Galler, R. and Chambers, T.J. (1989) Transcription of infectious yellow fever virus RNA from full-length cDNA templates produced by *in vitro* ligation. New Biol. *1*:285-296.

Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct the expression of cloned genes. Methods Enzymol. *185*:60-89.

E. Publications/presentations stemming from the contract work

Publications

1. Henchal, E.A., Narupiti, S., McAda. P., Fournier, M.J., Mason, T.L., Padmanabhan, R., Blok, J., Hoke, C. and Nisalak, A. (1986) Rapid detection of dengue and Japanese encephalitis viruses using nucleic acid hybridisation. *In*: Arbovirus Research in Australia: Proceedings Fourth Symposium. St. George, T.D., Kay, B.H. and Blok, J. (eds.) CSIRO Tropical Animal Science, pp. 112-118.

2. McAda, P.C., Mason, P.W., Schmaljohn, C.S., Dalrymple, J.M., Mason, T.L. and Fournier, M.J. (1987) Partial nucleotide sequence of the Japanese encephalitis virus genome. Virology *158*:348-360.

3. Mason, P.W., McAda, P.C., Dalrymple, J.M., Fournier, M.J. and Mason, T.L. (1987a) Expression of Japanese encephalitis virus antigens in *Escherichia coli*. Virology *158*:361-372.

4. Mason, P.W., McAda, P.C., Mason, T.L. and Fournier, M.J. (1987b) Sequence of the Dengue-1 virus genome in the region encoding the three structural proteins and the major nonstructural protein NS1. Virology *161*:262-267.

5. Mason, P.W., Dalrymple, J.M., Gentry, M.K., McCown, J.M., Hoke, C.H., Burke, D.S., Fournier, M.J. and Mason T.L. (1989) Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. Journal of General Virology *70:2037-2049*.

6. Huebner, R.C., Wysokenski, D., Mason, T.L. and Fournier, M.J. (1990) Expression of antigenically active Dengue-1 virus proteins using recombinant baculovirus. Vaccines 90: 125-130.

7. Mason, P.W., Zuegel, M.U., Semproni, A.R., Fournier, M.J. and Mason, T.L. (1990) The antigenic structure of Dengue Type 1 virus envelope and NS1 proteins expressed in *Escherichia coli*. Journal of General Virology *71*:2107-2114.

8. Mason, P.W., Pincus, S., Fournier, M.J., Mason, T.L., Shope, R.E. and Paoletti, E. (1991) Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. Virology *180*:294-305.

Meeting Presentations

1. Pang, T., Lam, S.K., Gunasegaran, K., Wong, S.Y., Fournier, M.J., Mason, T.L. and McAda, P.C. Rapid diagnosis of dengue virus infections. Proceeding of the First International Conference on the Impact of Viral Diseases on the Development of Asian Countries. 7-13 Dec., 1986; Bangkok, Thailand.

2. Mason, P.W., Fournier, M.J. and Mason, T.L. (1987). Immunological characterization of recombinant and authentic antigens of Japanese encephalitis virus. Scientific Meeting on New Approaches for the Development of Dengue Vaccines. World Health Organization. 7-8 Aug. 1987; Edmonton, Canada.

3. Mason, P.W., Fournier, M.J. and Mason, T.L. (1987). Immunological properties of Japanese encephalitis virus antigens expressed in *E. coli*. VII International Congress of Virology. 9-14 Aug., 1987; Edmonton, Canada. p.106.

4. Mason, P.W., Fournier, M.J. and Mason, T.L. (1987). Mapping epitopes in the E protein of Japanese encephalitis virus. VII International Congress of Virology. 9-14 Aug., 1987; Edmonton, Canada. p.288.

5. Mason, P.W., McAda, P.C., Fournier, M.J. and Mason, T.L. (1987). Structures of the genomes of the Japanese encephalitis and dengue-1 viruses and expression of the E and NS1 viral antigens in *E. coli*. Scientific Group on Development of Recombinant DNA Japanese Encephalitis and Dengue Fever Vaccines. World Health Organization. 3-4 Feb., 1987; Osaka, Japan.

6. Mason, P.W., McAda, P.C., Zuegel, M., Mason, T.L. and Fournier, M.J. (1987). Structure of the dengue-1 genome and expression of E and NS1 antigens in *E. coli*. VII International Congress of Virology. 9-14 Aug., 1987; Edmonton, Canada. p.106.

7. Pang, T., Lam, S.K., Koh, C.L., Gunasegaran, K., Chew, C.B, Wong, S.Y., Fournier, M.J., Mason, T.L. and McAda, P.C. (1987). Rapid diagnosis of dengue haemorrhagic fever. Proceedings of the 21st Malaysia-Singapore Congress of Medicine. Quek, D. (ed), pp. 43-45.

8. Zuegel, M.U., Mason P.W., Fournier, M.J. and Mason, T.L. (1987). Mapping epitopes on recombinant DEN-1 antigens. Scientific Meeting on New Approaches for the Development of Dengue Vaccines. World Health Organization. 7-8 Aug., 1987; Edmonton, Canada.

9. Mason, T.L. and Fournier, M.J. (1988). Flavivirus gene expression in bacterial cells. Development and Testing of Flavivirus Vaccines by the U.S. Army Medical Research and Development Command: Progress and Strategies. 1 Mar., 1988; Washington, D.C.

10. Fournier, M.J. and Mason, T.L. (1989). Expression of vaccine-candidate flavivirus proteins in *E. coli* and insect cell systems. World Health Organization Review on the Development of Dengue, Japanese and Other Flavivirus Vaccines. 24-25 June, 1989; Vienna, Austria.

11. Fan, W., Shope, R.E., Fonseca, B., Zuegel, M.U., Fournier, M.J., Mason, T.L. and Mason, P.W. (1989). Use of flavivirus-*E. coli* fusion proteins as diagnostic reagents to distinguish infections by different flaviviruses. The 38th Annual Meeting of the American Society of Tropical Medicine and Hygiene. 10-14 Dec., 1989; Honolulu, HA.

12. Huebner, R.C., Mason, P.W., Mason, T.L. and Fournier, M.J. (1990). Expression of antigenically active Dengue 1 virus proteins using recombinant baculoviruses. International Congress of Virology. Aug., 1990; Berlin, FRG.

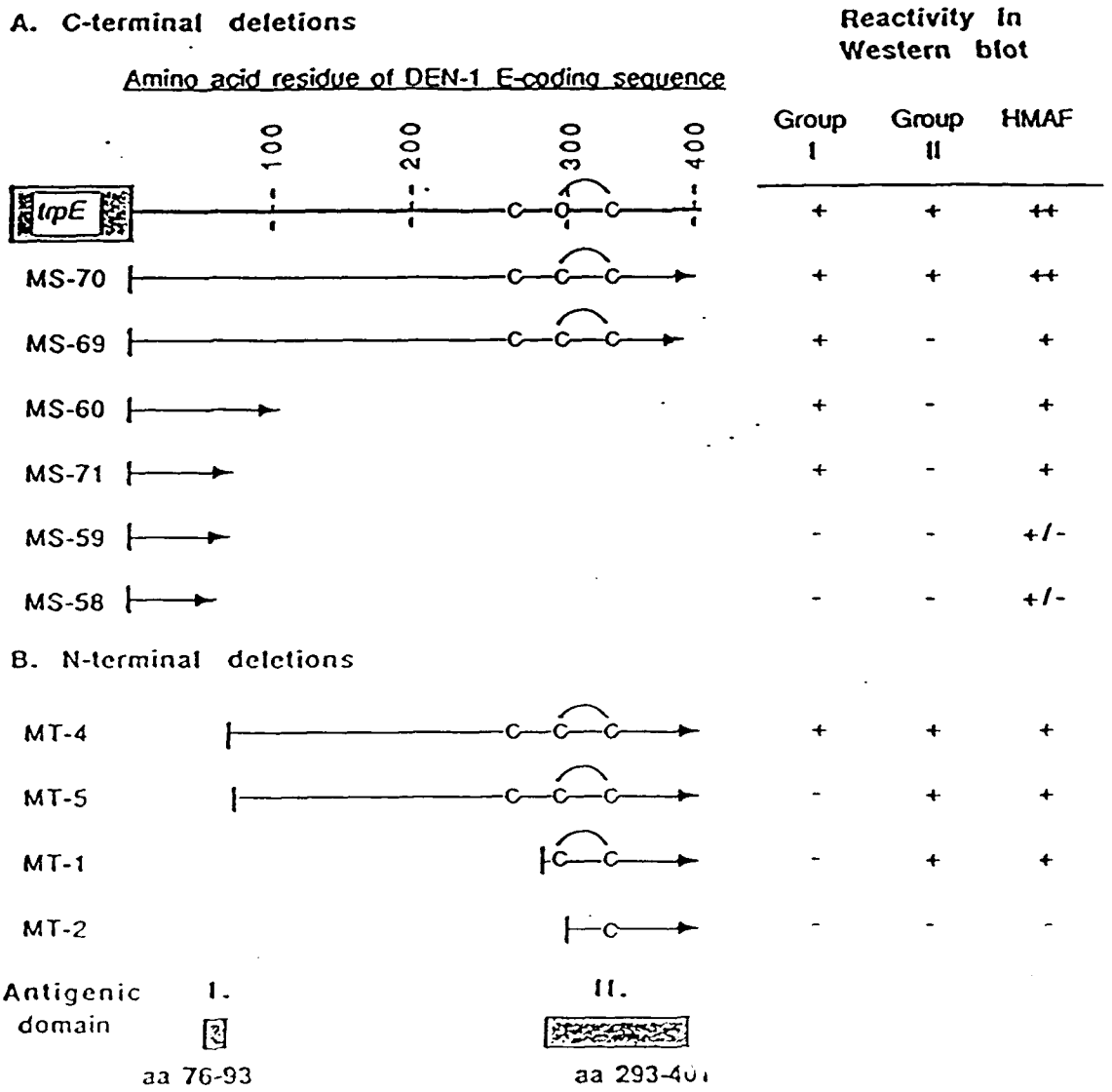

Fig.1. Summary of the deletion analysis used for mapping epitopes in the recombinant DEN-1 E protein. The clone designations for deletions extending from the carboxy terminus and the amino terminus, Figs 1A and 1B, respectively, are given on the left. The immunoreactivities of the derived recombinant proteins as determined by ELISA and western blot analyses are shown on the right. Group I and Group II antibodies are defined in Table I .

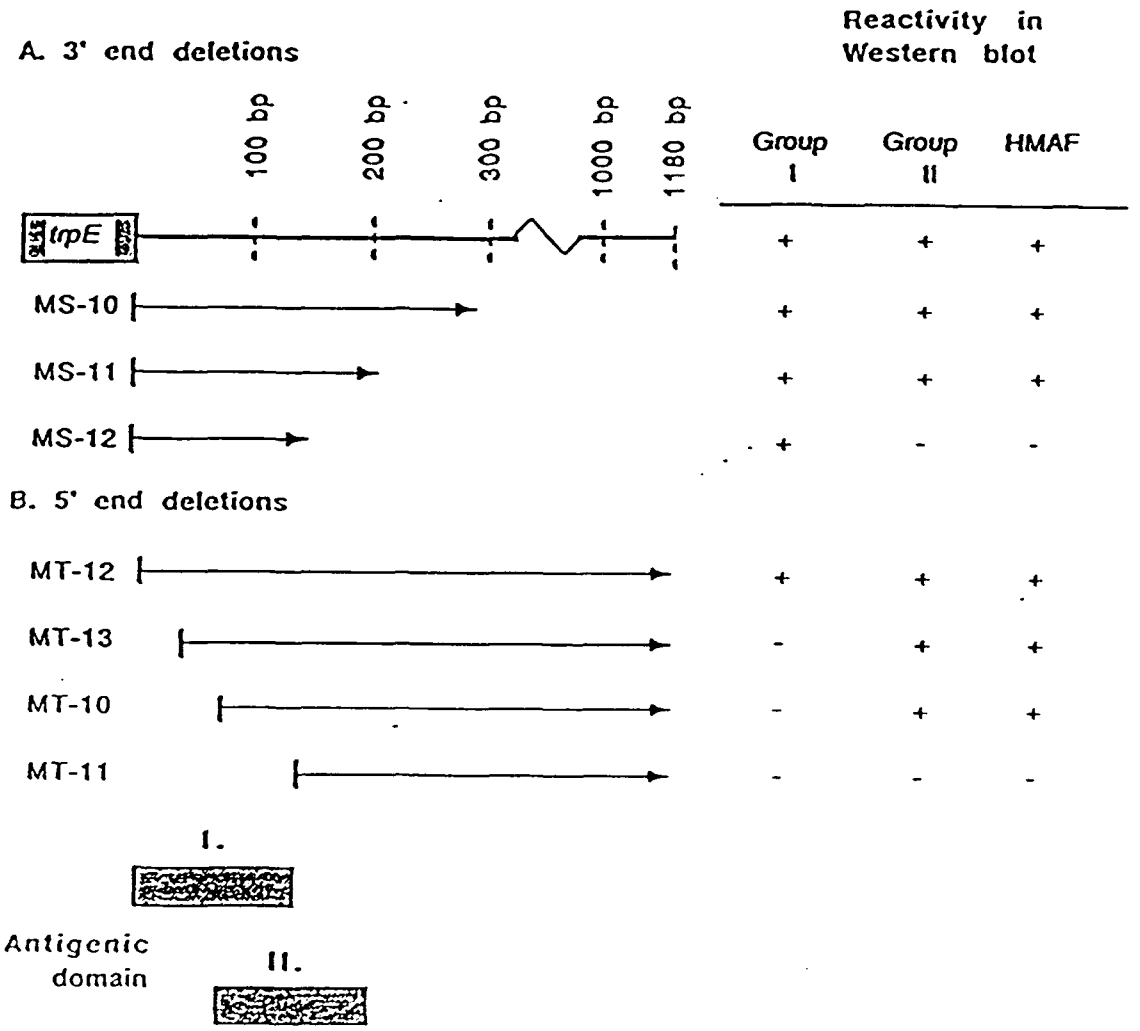

Fig.2. Summary of the deletion analysis used for mapping epitopes in the recombinant DEN-1 NS1 protein. The clone designations for deletions extending from the carboxy terminus and the amino terminus, Figs 2A and 2B, respectively, are given on the left. The immunoreactivities of the derived recombinant proteins as determined by ELISA and western blot analyses are shown on the right. Group I and Group II antibodies are defined in Table I .

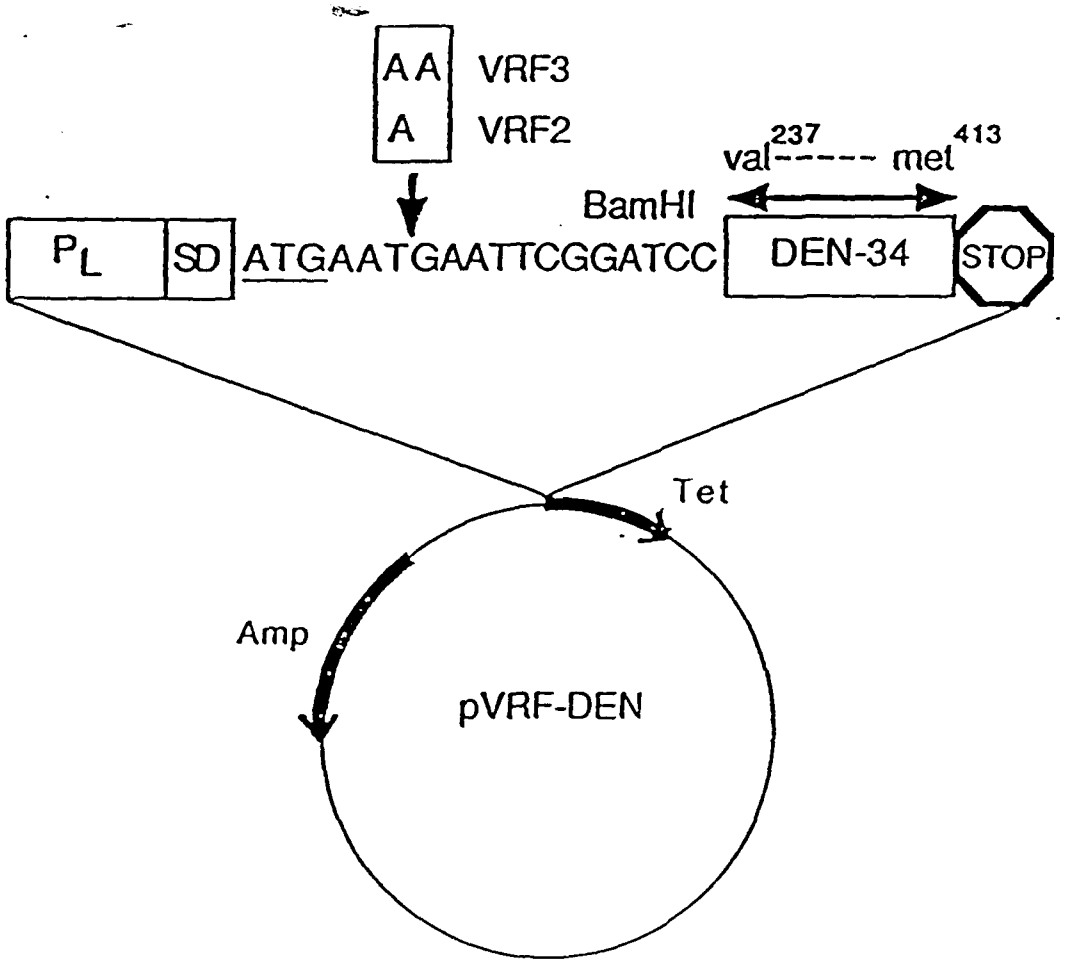

Fig.3 *E.coli* expression plasmid for production of non-fusion DEN1 antigens. The construction shown is for the antigenic domain of the E protein that we have previously shown to bind neutralizing and protective MAbs. Other DEN1 and JE sequences have also been ligated in this vector. Notice that three vectors are available (pVRF1, pVRF2, pVRF3), each corresponding to a different reading frame at the *Bam*HI cloning site. We have inserted a translational stop codon in each reading frame to insure proper termination at the end of the viral coding region. Expression from this plasmid is induced at 42°C in strains that carry a temperature sensitive phage λ repressor.

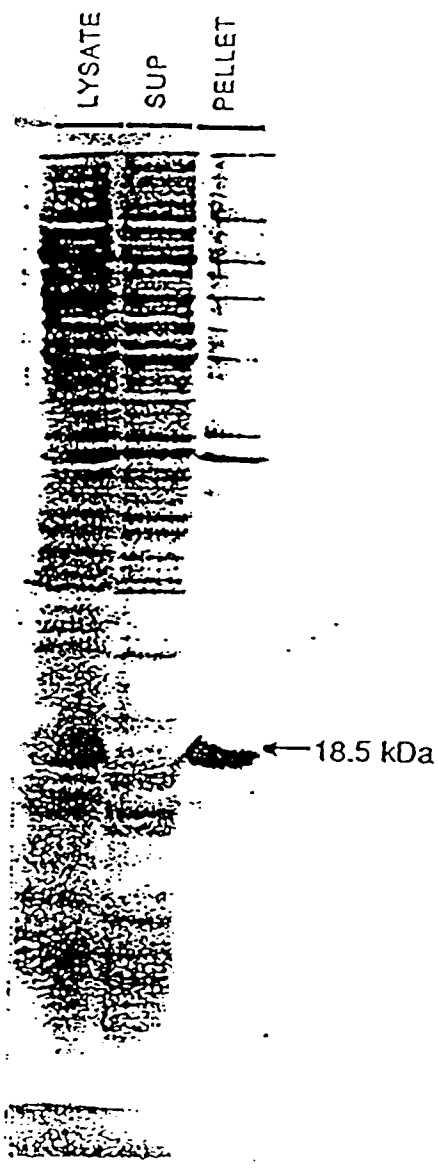

Fig.4. Expression of a DEN-1 E protein fragment in *E.coli*. *E.coli* cells (strain RR1) carrying the pVRF-DEN plasmid were grown at 30°C and then induced at 42°C for 2 hours. The cells were lysed and the lysate was fractionated into pellet and supernatant fractions by centrifugation at 110,000 x g for 90 min. Samples of the the lysate, supernatant and pellet fractions were solubilized in SDS-containing electrophoresis buffer and the polypeptides were resolved by electrophoresis on a 15% polyacrylamide gel. The figure shows the Commassie blue-stained gel. The DEN recombinant protein, indicated by the arrow, is quantitatively recovered in the pellet fraction.

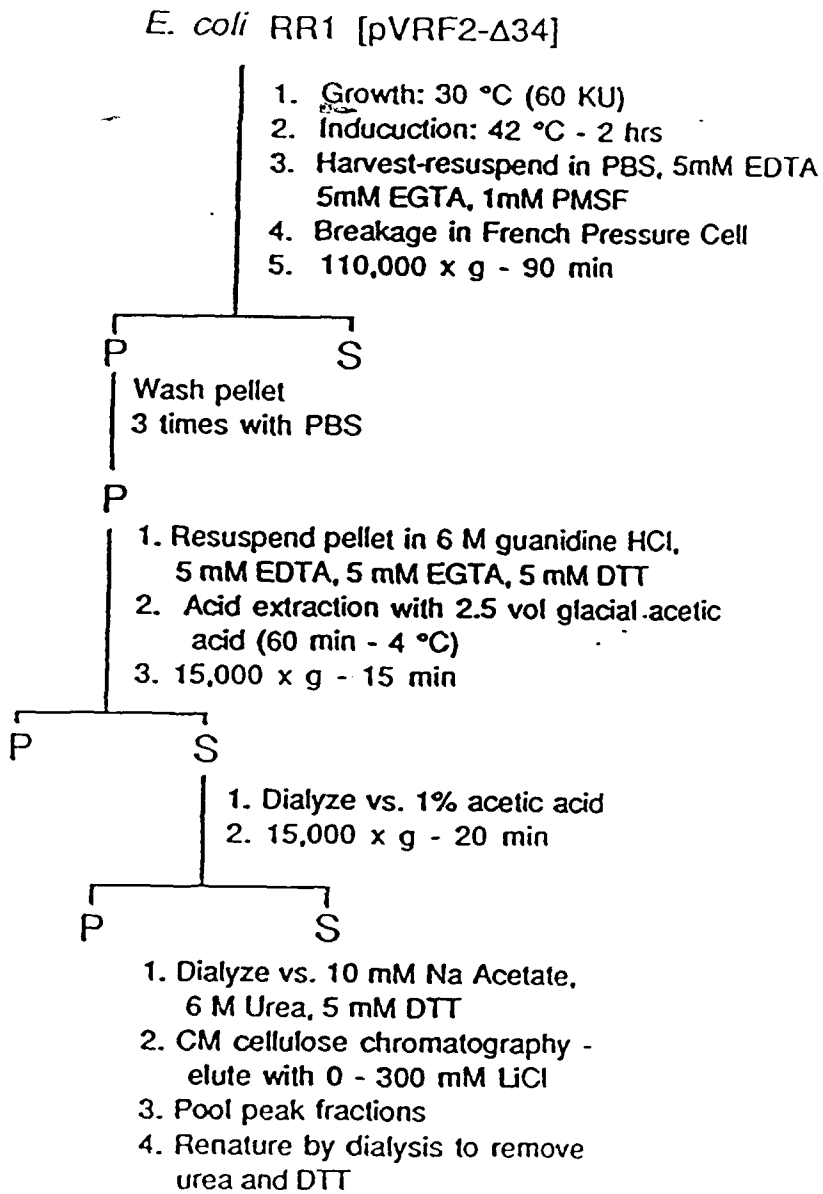

Fig. 5. Flow chart for the purification of the DEN1-Δ34 recombinant protein.

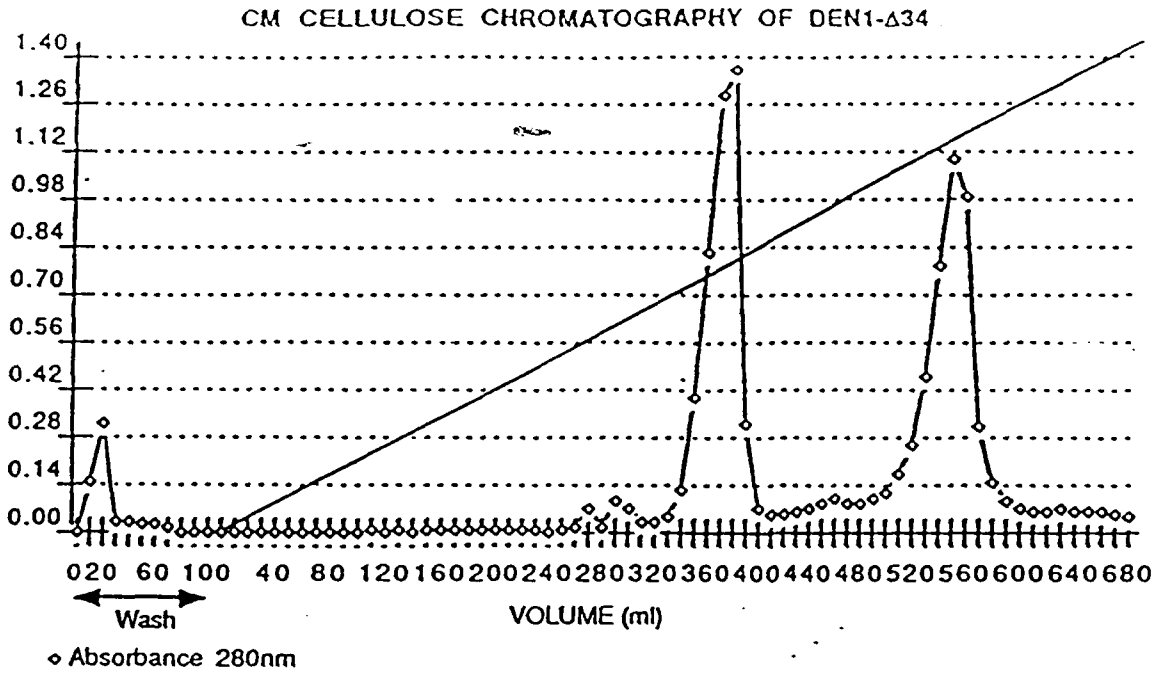

Fig. 6. CM-cellulose column chromatograpy in the purification of the DEN1-Δ34 protein. A 50 ml volume of the dialyzed, acid-extracted protein fraction (see Fig. 5) in 10 mM sodium acetate, pH 5.5, 8 M urea, 5 mM DTT was applied to a 2.5 x 40 cm column containing CM-cellulose equilibrated in the same buffer. The column was washed with 100 ml of the sample buffer and eluted with a 0-300 mM LiCl salt gradient. The second peak of UV-absorbing material contains the DEN1-Δ34 protein.

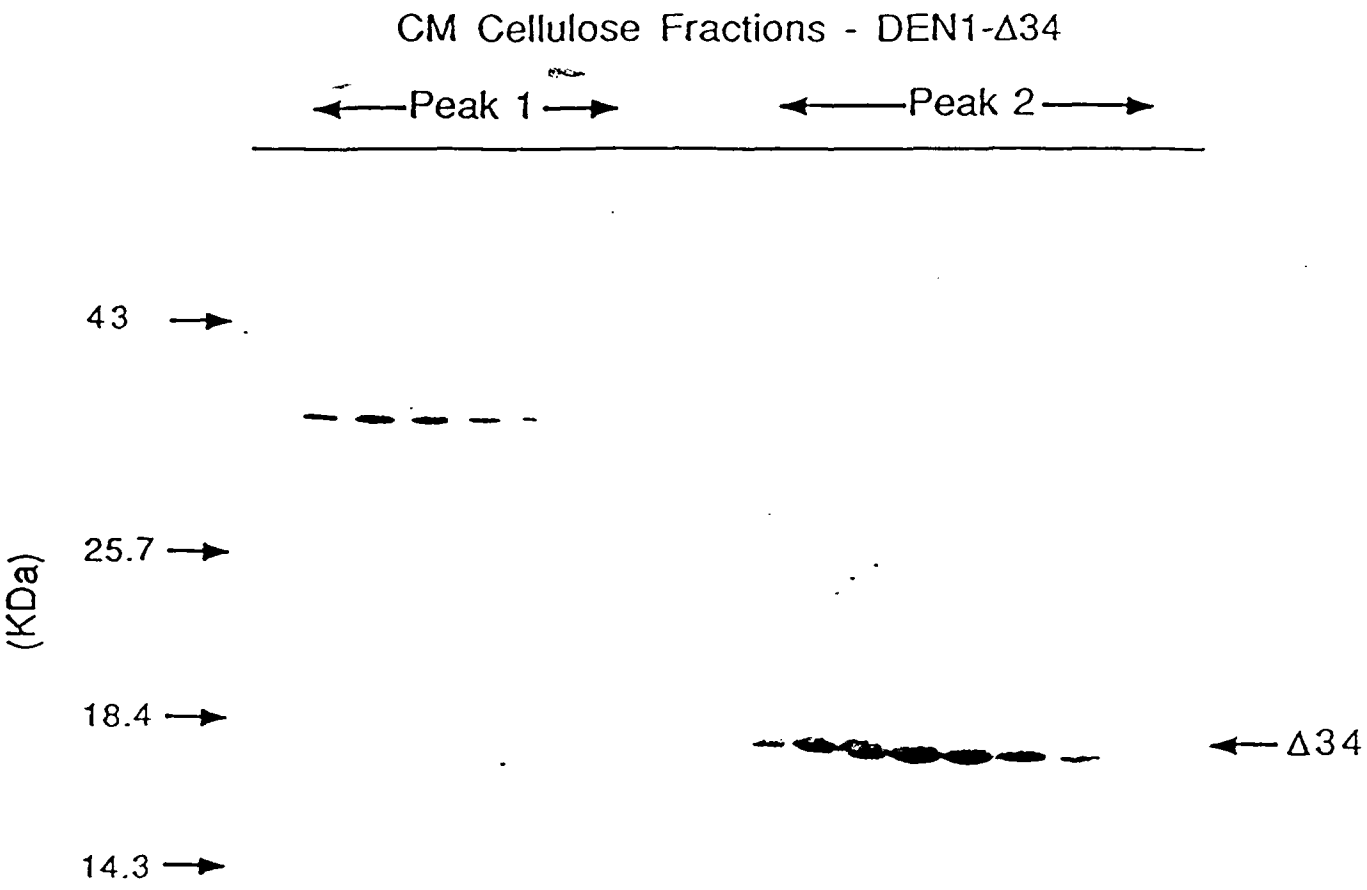

Fig. 7. Polyacrylamide gel electrophoresis of proteins in the peak fractions from CM-cellulose column chromatography. 20 μl samples were taken from fractions from across the first and second peaks (Fig.6) and mixed SDS-sample buffer. The proteins were resolved by electrophoresis in a 15% polyacrylamide gel and visualized by staining with Commassie blue.

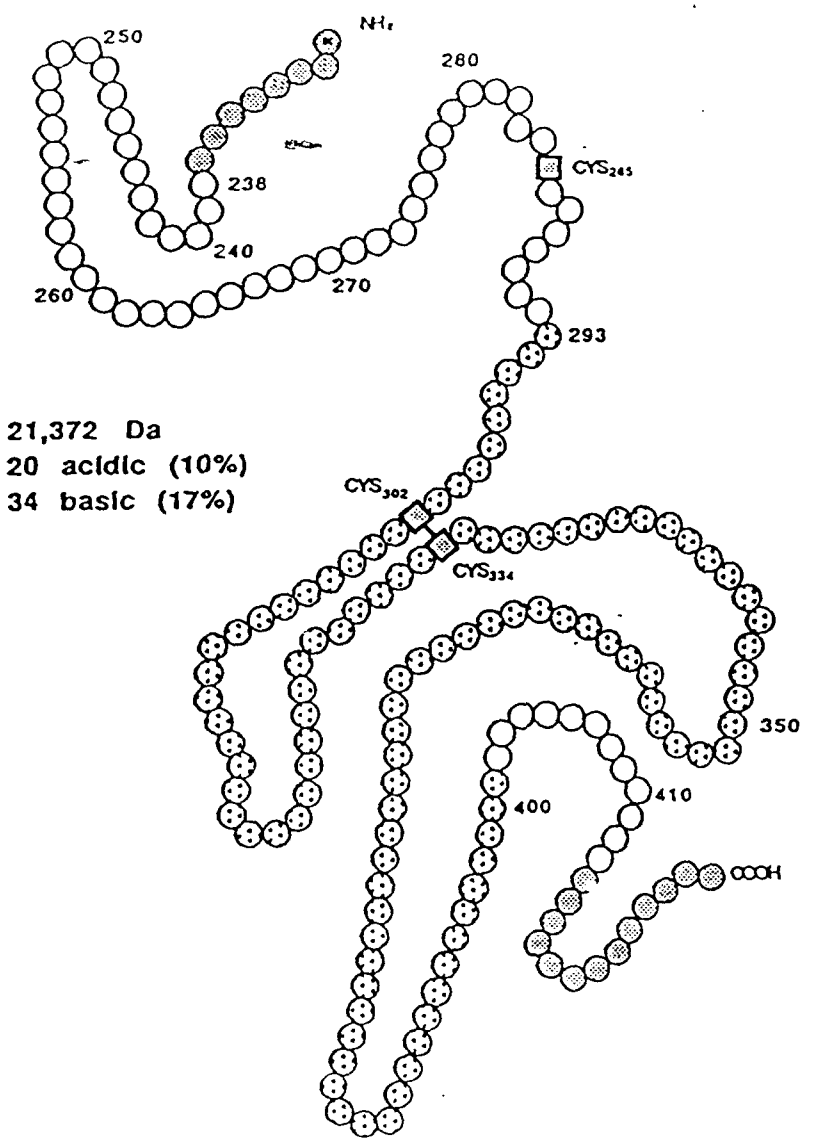

Fig. 8. Schematic representation of the DEN1-Δ34 recombinant protein. The dark circles represent nonviral amino acids encoded by vector sequences in the cloning site. The stippled circles depict the DEN-1 domain II antigenic region defined by deletion analysis (see Fig.1 for details).

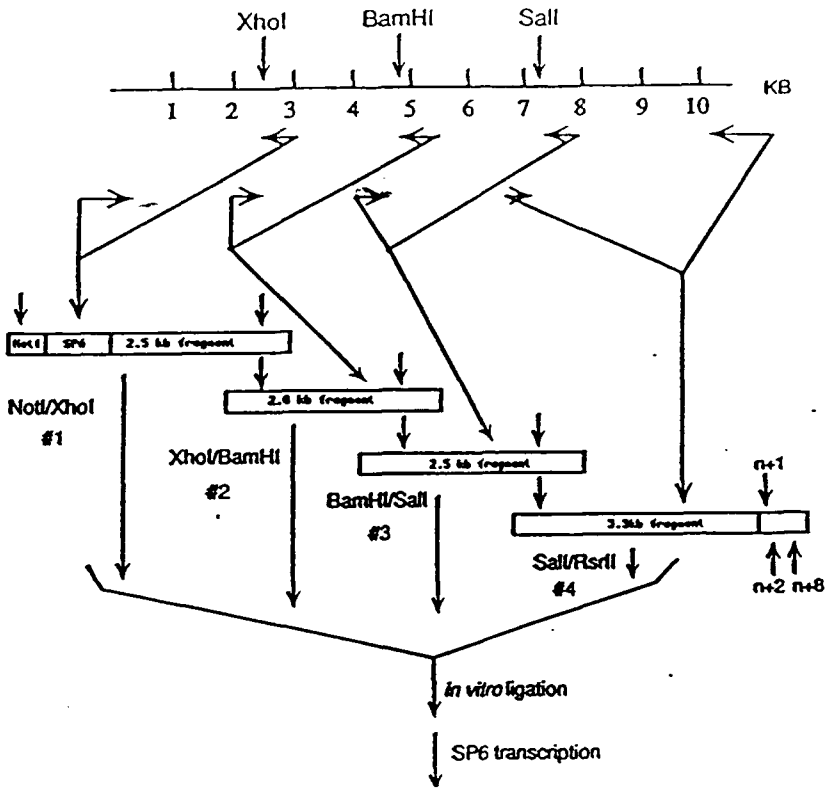

Figure 9. Strategy for the construction of D-1 cDNA by PCR. Relative positions of sense and antisense primers (arrows pointing right or left respectively), are shown for first and second strand synthesis and PCR amplification. Vertical arrows indicate relevant restriction sites that will be used for *in vitro* ligation of amplified segments (open boxes). Production of a recombinant D-1 RNA by transcription from the SP6 promoter is indicated. Each amplified fragment is denoted by a number (1-4) where: #1 is the NotI/XhoI fragment; #2, the XhoI/BamHI fragment; #3, BamHI/Sal I and #4, Sal I/RsrII. Primers designed to amplify each fragment are designated with the fragment number and either S or A for sense or antisense respectively. For example, fragment #1 (NotI/XhoI) is amplified by primers 1S and 1A corresponding to the S1NotI and A2Xho 2.6 primers shown in Fig.1. The full-length *in vitro* ligated cDNA will contain: 1) one extra nucleotide (n+1) after ribozyme digestion of the D-1 transcript; 2) two extra nucleotides (n+2) after RsrII digestion of the template; or 3) eight extra nucleotides (n+8) following digestion of cDNA with SpeI. n, full-length D-1 cDNA; kb, kilobases.